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(54) Title: NEW METHOD OF PRODUCING CELL CULTURES

(57) Abstract: A method for producing a cell culture line, and more specifically a method which allows the production of a non-insect invertebrate cell culture line by manipulating the expression of a gene or genes involved in cell cycle control.

New Method of Producing Cell Cultures

The present invention relates to a method for producing a cell culture line, and more specifically a method which allows the production of a non-insect invertebrate cell culture line.

The routine maintenance of mammalian cell lines has enabled huge advances to be made in the study of cell signalling, and also in the production of medically important compounds. Cell lines from other sources are also now in regular use, which provide novel approaches to compound production, as well their own specific applications in research. In particular, insect cell lines are available for this type of work, and have several advantages over mammalian cell cultures in terms of recombinant expression systems, as they are cheaper, tolerant of variable cell culture conditions and have high expression levels. This illustrates the benefits of diversification in cell culture approaches.

Despite the advantages of having multiple in vitro based systems for research and recombinant compound production,

cell cultures from invertebrates other than insects are still not available. Primary cultures from sponges, molluscs, crustaceans, echinoderms, ascidians and nematodes have been established and differentiation has been observed. However, few cultures have shown sufficient proliferation to allow passaging of cell lines, and techniques that have been employed in mammalian and insect cells, such as viral transfection and exact recreation Of *in vivo* conditions, have not successfully translated into non-insect invertebrate cell cultures. This means the key goals of long-term sustainability and reproducibility have not been achieved, and remain major obstacles in the exploitation and production of non-insect invertebrate cell cultures.

It can be seen that it would be extremely beneficial for us to be able to provide a sustainable and reproducible non-insect invertebrate cell culture.

It is an object of the present invention to provide an improved method of producing cell cultures.

Throughout this document, RNAi should be taken to mean RNA interference (which has alternatively been referred to as post-transcriptional gene silencing).

According to a first aspect of the present invention, there is provided a method of culturing cells, including the step of inhibiting the expression of a gene or genes involved in cell cycle control.

Preferably the gene or genes are blocked.

Preferably RNAi is used to block the expression of genes involved in cell cycle control.

Preferably double stranded RNA is used to block the expression of genes involved in cell cycle control.

Preferably the *retinoblastoma (Rb)* gene is blocked.

Optionally the *P53* gene is blocked.

A further option is that a number of cell cycle control genes are blocked.

Alternatively, a gene equivalent to the *retinoblastoma (Rb)* gene will be blocked.

Preferably the equivalent gene will encode the same protein (allowing for genetic code redundancy).

Optionally the equivalent gene will encode for a protein that has the same function as retinoblastoma.

A further option is that the equivalent gene will show >35% sequence homology with the original blastoma gene.

Preferably the equivalent gene will show >99% homology.

Preferably the method further comprises the step of immortalising the cells using transfection techniques.

Preferably the transfection technique used is the inclusion of a gene sequence coding that native telomerase reserve transcriptase enzyme (TERT).

In order to provide a better understanding of the present invention, we will now describe embodiments by way of example only, and with reference to the following drawings, in which:

Figure 1 shows a schematic of the proposed approach; and

Figure 2 shows components of the Rb pathway (adapted from Classon, M., Harlow, E. 2002. Nature Reviews 2: 910-917)

Nematode cells have been difficult to culture, although there has been reports of primary cell culture systems for *C. elegans* embryonic cells which, although unable to divide, where able to differentiate into neurons and muscle cells (Christensen et al 2002). The inventors in the present case have been able to repeat the cell isolation and culture procedures detailed in the study using both *C. elegans* and *Aphelencus avenae*, suggesting that it is a viable starting point for cell culture manipulations. The inventor has also succeeded in maintaining ascidian and echinoderm cells in vitro under a number of different conditions with the same observed results. That is, cells remain viable for several weeks in vitro but there is no evidence of growth. This can work as an initial point for the establishment and maintenance of cell cultures.

Manipulation of the Cell Cycle

Once cell cultures are established, they can be manipulated to promote proliferation. The lack of identified native non-insect invertebrate viruses, lack of obvious tumours and the low levels of proliferation in

dissociated cells means that approaches developed in mammalian systems or insect systems are not easily translated into non-insect invertebrates. In this case, it has been found that RNAi can be used for the blocking of gene expression to overcome some of the obstacles to genetic manipulation of non-insect invertebrate cells. RNAi is a conserved biological response to double stranded RNA, and is known variously as RNA interference (RNAi) or post-transcriptional gene silencing. Double stranded RNA corresponding to a gene or a coding region of interest is introduced into an organism, resulting in the degradation of the corresponding mRNA.

RNA interference (RNAi) is a cellular mechanism to regulate the expression of genes and the replication of viruses. This mechanism is mediated by double-stranded small interfering RNA molecules (siRNA). RNAi technology is a comparatively recent discovery believed by scientists to constitute an important aspect of a cell's natural defensive mechanism against parasitic viruses. Critically, the cell responds to a foreign (double stranded) form of siRNA introduced into the cell by destroying all internal mRNA with the same sequence as the siRNA.

RNAi has been used extensively for gene expression studies in C. elegans, and it has been found that double stranded RNA can easily be taken up by whole animals from the culture media or by feeding. This results in a specific gene silencing effect. The effect has also been demonstrated in *C. elegans, Drosophila* and *Anopheles* cell cultures. In the preferred embodiment, the RNAi approach will be based on the direct inhibition of cell cycle

control genes, rather than the stimulation of cell cycle, as has been the case in all previous transfection studies. This presents a novel method of inducing cell cycle activity. The key cell cycle control gene retinoblastoma (Rb) is the preferred target in this embodiment. The Rb protein acts as the check point for progression into "S" phase of the cell cycle and is relatively well conserved.

The approach described by Caplen et al 2000 and Christensen et al 2002 (Christensen, M., Estevez, A., Yin, XY., Fox, R., Morrison, R., McDonnell, M., Gleason, C., Miller, DM., Strange, K. 2002. Neuron, 33: 503-514; Caplen, NJ., Fleenor, J., Fire, A., Morgan, RA. 2000 Gene 252: 95-105) can be applied to primary cell cultures. In the preferred embodiment, this will be to primary cell cultures of non-insect invertebrates. A native gene equivalent to *Rb* will be identified and confirmed by PCR and sequencing. Gene equivalents include those genes which show structural homology, those which show sequence homology, those genes which encode for the same protein but allow for genetic code redundancy, and those genes which encode for proteins that have the same function as retinoblastoma. Typically when considering structural or sequence homology, we would be looking for homology in the region of 25% to 99%.

Preferably it would be in the region of 50% to 99%.

Most preferably it would be in the region of 75% to 99%.

Once the target sequence has been identified, double stranded RNA is designed to deactivate mRNAs encoding

native Rb like protein. The whole embryo and cell culture approaches will be taken by incubating embryos or cells with the specific double stranded RNA. If viable, the embryos will also be dissociated to form primary cultures. Consumation of effectiveness of the RNAi can be measured by measurement of the mRNA levels of the target gene using northern blotting or equivalent.

In alternative embodiments, RNAi can be carried out on the *P53* gene and other cell cycle control genes. As well as being carried out individually, RNAi can be carried out on multiple cell cycle control genes at the one time.

Expressing Genes Which Induce Cell Proliferation

Cells that have been stimulated to proliferate using the RNAi approach may still be limited in a number of divisions they can undergo. To produce cell lines for long-term use, immortilisation of the cells using transfection techniques may be required in certain cases. One method for the promotion of cell division, which has not yet been attempted in invertebrate cells, is the enhanced expression of the telomerase reverse transcriptase enzyme (TERT), an approach which has been developed in mammalian cell culture systems, but is typically not considered for invertebrate systems. The TERT gene is highly conserved and it has been identified in the species including *Arabidopsis thaliana, Giardia lamblia* and *C. elegans*. Corresponding native TERT genes will be identified from databases or by targeting conserved regions using PCR and sequencing for whichever target species is of interest (gene equivalents will be considered as described previously). These can then be used to design novel constructs for cell transfection.

Native promoters identified from the literature and promoters shown to have cross species activity, such as that of the *Drosophila hsp*70, will be used to build vectors containing the native TERT sequence. If possible, a reporter system (e.g., GFP), will also be incorporated into the vector to confirm gene expression.

Although introduction of gene constructs has been attempted in some invertebrates, using a mixture of native and non-native promoters and pan-tropic viruses, in this case non-viral methods of DNA introduction, such as lipofection, injection or electroporation will be used as they have no host-specific requirement and are less likely to produce an immunological response.

Protocol details

Primary cultures of non-insect invertebrate tissues and cells can be generated by excision of the target tissue from the organisms, or collection of blood cells. If necessary, tissue can be dissociated mechanically or chemically to generate cell cultures. A range of culture conditions using commercial and in-house media formulations can be used.

Specific double-stranded RNA (dsRNA) for relevant gene products (as indicated) can be generated by any currently available protocols including *in vitro* transcription, enzymic digestion of larger RNA molecules, direct expression of siRNA molecules from a plasmid, synthetic construction of siRNA.

The dsRNA may be introduced into the cells or tissues under examination by several protocols including incubation in the growth media, lipofection, injection, use of plasmids which encode specific siRNAs which will then be produced and processed inside the target cells = DNA-directed RNAi (these plasmids may be introduced into the target cells by lipofection, injection or electroporation).

The success of the gene silencing can be assessed by RNA extraction of the target cells and tissues, and then by Northern blotting or Real-time RT-PCR. The use of controls expressing a reporter system such as GFP may indicate the success of the technique. Also, silencing of housekeeping genes (for example those encoding actin) can be investigated to check the specificity of the effect. The success of the effect will also be monitored by microscopic examination of the cells, cell counts, assessment of metabolic activity and monitoring of cell cycle activity (eg by bromodeoxyuridine incorporation), to monitor cell growth and viability. Cultures showing cell proliferation can be passaged and fresh dsRNA added. Cells may be cryostored and resuscitated using standard techniques.

Similarly, cell lines may be created by the expression of a native telomerase reverse transcriptase enzyme. The native gene encoding this enzyme in the target species can be incorporated into a DNA vector, which includes a promoter to enhance expression. Cultures can be created as indicated above. DNA vectors can be introduced into cells using lipofection, injection, electroporation or any novel techniques. Gene expression can be monitored

using a gene reporter system such as GFP or luciferase, and assessment of cell cycle activity as in point 5 above.

It can be seen that the methods described allow the production of a non-insect invertebrate cell line.

CLAIMS

1. A method of culturing cells, including the step of inhibiting the expression of a gene or genes involved in cell cycle control.

2. A method of culturing cells, as claimed in Claim 1, wherein the cells are non-insect invertebrate cells.

3. A method of culturing cells, as claimed in Claim 1 or 2, wherein the gene or genes are blocked.

4. A method of culturing cells, as claimed in any of the previous claims, wherein RNAi is used to block the expression of genes involved in cell cycle control.

5. A method of culturing cells, as claimed in claims 1 to 3, wherein double stranded RNA is used to block the expression of genes involved in cell cycle control.

6. A method of culturing cells, as claimed in any of the previous claims, wherein the *retinoblastoma (Rb)* gene is blocked.

7. A method of culturing cells, as claimed in any of the previous claims, wherein the *P53* gene is blocked.

8. A method of culturing cells, as claimed in any of the previous claims, wherein a number of cell cycle control genes are blocked.

9. A method of culturing cells, as claimed in any of the previous claims, wherein, a gene equivalent to the *retinoblastoma (Rb)* gene will be blocked.

10. A method of culturing cells, as claimed in claim 9, wherein the equivalent gene will encode the same protein (allowing for genetic code redundancy).

11. A method of culturing cells, as claimed in claim 9 or 10, wherein the equivalent gene will encode for a protein that has the same function as retinoblastoma.

12. A method of culturing cells, as claimed in claims 9 to 11, wherein the equivalent gene will show >35% sequence homology with the original blastoma gene.

13. A method of culturing cells, as claimed in claims 9 to 11, wherein the equivalent gene will show >99% homology.

14. A method of culturing cells, as claimed in any of the previous claims, wherein the method further comprises the step of immortalising the cells using transfection techniques.

15. A method of culturing cells, as claimed in Claim 14 wherein the transfection technique used is the inclusion of a gene sequence coding that native telomerase reserve transcriptase enzyme (TERT).

Schematic of
proposed approach

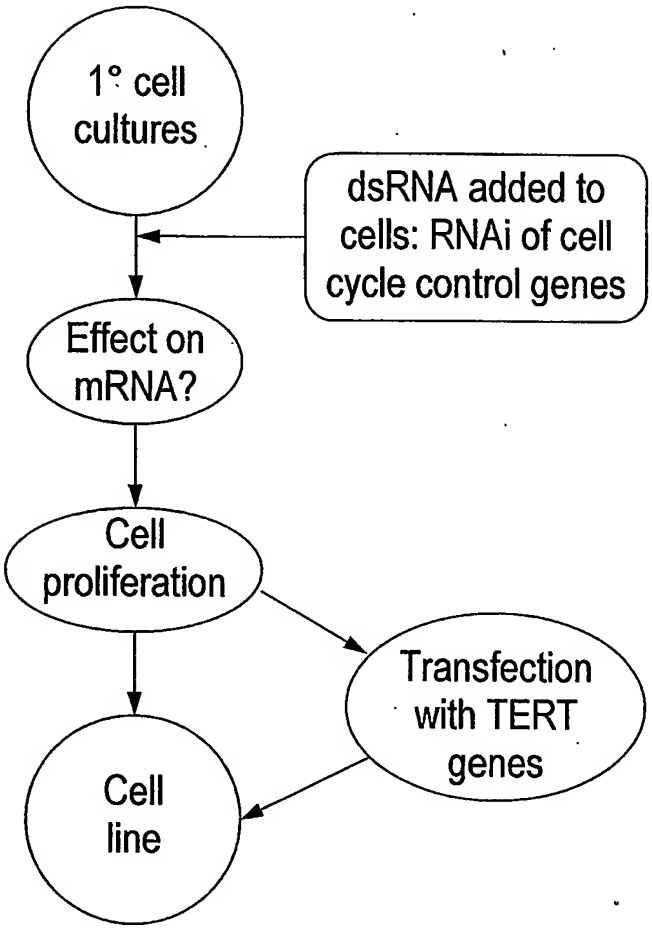

FIG. 1

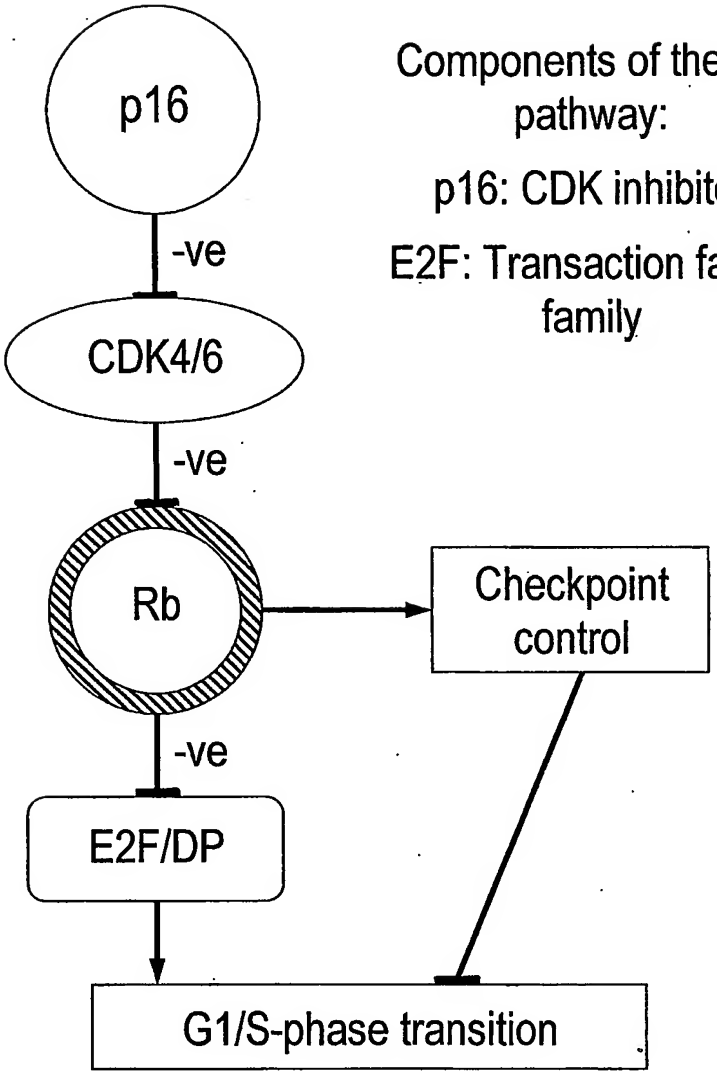
p16
-ve
CDK4/6
-ve
Rb
-ve
E2F/DP
Checkpoint control
G1/S-phase transition
Components of the Rb pathway:
p16: CDK inhibitor
E2F: Transaction factor family

FIG. 2